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Research Article

Luciferase a light source for the silica-based optical waveguides (spicules) in the demosponge *Suberites domuncula*

W. E. G. Müller[a,*], M. Kasueske[a], X. Wang[b], H. C. Schröder[a], Y. Wang[b], D. Pisignano[c] and M. Wiens[a]

[a] Institut für Physiologische Chemie, Abteilung Angewandte Molekularbiologie, Universität, Duesbergweg 6, D-55099 Mainz (Germany), Fax: +49-6131-39-25243, e-mail: wmueller@uni-mainz.de
[b] National Research Center for Geoanalysis, 26 Baiwanzhuang Dajie, CHN-100037 Beijing (China)
[c] National Nanotechnology Laboratory, Istituto Nazionale di Fisica della Materia-Consiglio Nazionale Delle Ricerche, Università degli Studi del Salento, Via Arnesano, I-73100 Lecce (Italy)

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Abstract. Two classes of sponges (animal phylum Porifera) possess a siliceous skeleton which is composed of spicules. Studying the optical fiber-mechanical properties of large spicules from hexactinellid sponges (> 5 cm) it was demonstrated that they are effective light-collecting optical fibers. Here, we report that the demosponge *Suberites domuncula* is provided with a biosensor system composed of the (organic) light producing luciferase and the (inorganic) light transducing silica spicules. The light transmission feature of these smaller spicules (200 μm) has been demonstrated and the ability of sponge tissue to generate light has been proven. Screening for a luciferase gene in *S. domuncula* was successful; the recombinant luciferase was prepared and shown to be bioactive. The luciferase protein is abundantly present in the close neighborhood of the spicules. The expression of the *luciferase* gene is under the control of light.

Keywords. Sponges, *Suberites domuncula,* optical waveguide, luciferase, luciferin-regenerating enzyme, spicules.

Introduction

Sponges (phylum Porifera) represent a successful animal taxon that evolved prior to the Ediacaran-Cambrian boundary (542 million years ago (MYA) [1]. They have developed almost the complete array of cell- and tissue-based interaction systems, allowing the establishment of a functional, multicellular body [2]; however, one cell/tissue-communication system is missing in sponges, the network of neurons [3]. This fact is puzzling and enigmatic, since these animals indeed possess receptors, known to be involved in the nervous system in evolutionary younger animal phyla. As an example, the metabotropic glutamate/GABA-like receptor has been identified and cloned from *Geodia cydonium* [4]; members of that receptor family are involved in the vertebrate nervous system [5]. It is also well established that sponges, some of which grow taller than 1 m, can react to stimuli with coordinated contractions [6]. It had already been speculated that an alternative inter-cellular signaling system might exist in demosponges, e.g. in *Tethya seychellensis*, which substitutes for a neuron-based communication system. Such a system might be considered to rely on the spicular network [7]

* Corresponding author.

consisting of a light generating system (e.g. luciferase), a light-guiding system (e.g. siliceous spicules), and receiver cells (e.g. neuronal glutamate/GABA-like receptors).

The two phylogenetically oldest classes of sponges, the Demospongiae and the Hexactinellida, are distinguished from the younger class of Calcarea by their siliceous skeleton. Their skeletal elements are termed spicules and comprise species-specific morphologies and arrangements. To support the assumption that the spicules may act as natural "pipelines for light" [7] also in nature, within the sponges, the optical fiber-mechanical properties of the spicules had to be studied. In experiments with spicules from hexactinellids, using *Rosella racovitzae* [8, 9], some optophysical properties have been elucidated; it had been identified that they function as optical waveguides. In addition, it could be determined, through three-point bend tests, that both the elastic modulus and the nanohardness values of the spicules are about half that of either fused silica or of commercial glass optical fibers [9]. These studies were extended with the hexactinellid *Euplectella aspergillum* by the demonstration that the basalia spicules display specific compositional variations within the glass-organic composite and that they can function as single-mode, few-mode, or even multimode fibers [10]. Taking advantage of the large size of siliceous spicules from some hexactinellids – more than 100 mm in length – additional studies have been performed using the giant spicules (the 40–120 mm long stalk spicules-basalia) from *Hyalonema sieboldi* [11] and the giant basal spicules (3000 mm long) from *Monorhaphis* [12]. The data gathered showed that the spicules, composed of up to 800 concentrically arranged silica lamellae, function as optical glass fibers with special features of light transmission, e.g. (*i*) only wavelengths between 615 and 1310 nm can pass through the fibers and (*ii*) light below wavelengths of 615 nm and above 1310 nm is completely cut off. In turn, it had been hypothesized that the spicules/siliceous fibers might be involved in these animals in a photoreception-like system [11]. To further substantiate this assumption, first genes had been cloned also from hexactinellids [12]. As an example, a gene coding for a photolyase-related protein from *Aphrocallistes vastus* had been identified that might be a candidate for light reception, preferentially around the violet/blue end of the spectrum. However, one molecule essential to underpin the view that spicules act also within the body of the sponges as waveguides for the transmission of light/electrical and/or chemical signals, similar to a neuron-like network system, needs to be identified: A luciferase(-related) enzyme. Up to the present study a light generating enzyme, a luciferase (or a luciferase-like molecule), had not been identified in sponges.

Bioluminescence, a chemiluminescence reaction, is a widespread phenomenon in nature, a process during which two molecules react in an organism with the emission of light. Intensive research started with the elucidation of the luciferin-luciferase reaction in the firefly *Photinus pyralis* [13]. Soon, it was recognized that luminescence is also found in bacteria, fungi, dinoflagellates, radiolarians and Metazoa [14]; most of these organisms inhabit the marine environment [reviewed in: 15, 16]. Later, molecular biological studies revealed that luciferase is a generic name and includes a series of different classes of luciferase molecules. Luciferin has been identified in bacteria (a reduced riboflavin phosphate ($FMNH_2$) that undergoes oxidation by luciferase in association with long-chain aldehyde and an oxygen molecule(s)), in dinoflagellates (it is conformationally shielded from luciferase at the basic pH of 8 but is set free and accessible to oxidation at the more acidic pH of 6), in the marine ostracod *Vargula* (it is acquired by ingestion of bacteria), in coelenterates (its activity is controlled by the concentration of Ca^{2+} and the molecule shares homology with the calcium-binding protein calmodulin) and in insects (fireflies, glowworms and click beetles) (luciferin, a benzothiazole, that in fireflies (*Photinus* or *Luciola*) has the unique property of requiring ATP as a co-factor to convert into an active molecule); [reviewed in: 15, 16]. The corresponding luciferase sequences from the different taxa have been cloned and analyzed, e.g. from bacteria of the genera *Photobacterium* and *Vibrio*, from the firefly (*Photinus*), from the jellyfish *Aequorea*, from the marine ostracod *Vargula*, from the deep-sea shrimp *Oplophorus*, and the anthozoan sea pansy *Renilla reniformis*; they had been found to be dissimilar between the different organismic taxa [reviewed in: 15–17].

For the verification that luciferase exists in sponges, the demosponge *Suberites domuncula* had been used, since its genome is the best understood sponge model to date, both with respect to the number of genes and to their functions [reviewed in: 2]. The siliceous spicules of the demosponges are usually smaller (< 200 μm) than those of the hexactinellids; however they have the same chemical composition as the hexactinellid spicules [18, 19].

In general, the emission spectra of the bioluminescence system are in the blue-green range [14] and vary in the firefly luciferase/luciferin system in the range of 520 nm to 620 nm [20, 21]. This range fits perfectly with the experimental data determined by us previously. Using the spicules from *H. sieboldi* we measured that they act as optical fibers with a lower wavelength cut-off edge at 615 nm within the broad

spectrum of white light [11]. The newly discovered *S. domuncula* luciferase sequence belongs to the group of the firefly luciferases, and hence might, as can be expected, represent a bifunctional enzyme, catalyzing both the luminescence reactions and the synthesis of long-chain fatty acyl-CoA synthetase [22]. In the broader sense this enzyme can be grouped to the 4-coumarate:CoA ligases, existing in plants [23, 24].

In order to identify the luciferase in the sponge *S. domuncula* our EST database was screened with the main focus on the presence of polypeptides, comprising the characteristic domains for metazoan luciferase [25, 26], e.g. the acetyl-coenzyme A (acyl-CoA) synthetase region and the luciferin-binding site residues. The database, which comprises 30 000 ESTs, can be considered to cover most of the genes in these animals [27]. Indeed, one fragment was found which shared high sequence similarity to the firefly luciferase.

The firefly luciferase converts the substrate luciferin, a heterocyclic carboxylic acid, and ATP into the corresponding luciferyl adenylate, and (with the consumption of molecular oxygen) to an electronically excited-state product, which emits a photon of visible light [see: 28]. The binding site for luciferin to the luciferase from *P. pyralis* had been studied in detail [29]. Data from Zako et al. [30], Nakatsu et al. [29], and Tafreshi et al. [31] showed that the dehydroluciferin moiety interacts in the hydrophobic pocket of the luciferase from *Luciola cruciata* with the regions, consisting of α-8 (amino acid residues 246–258), β-12 (284–287), β-13 (311–314), β-14 (337–340), β-15 (349–351) and α-loop (341–348).

During the flashing reaction luciferin is converted by the monooxygenase luciferase to oxyluciferin [see: 32]. In turn, oxyluciferin displays a strong inhibitory effect on the firefly luciferase reaction in a competitive manner [33]. Hence, intensive screening for luciferin-regenerating system(s) started [34, 35] and resulted in the identification and molecular cloning of a luciferin-regenerating enzyme from *P. pyralis* and related insects [32, 36, 37]. This enzyme converts oxyluciferin in the presence of D-cysteine to the intermediate 2-cyano-6-hydroxybenzothiazole [32]. Phylogenetic analyses revealed that the luciferin-regenerating enzyme shows similarity to senescence marker protein-30 (SMP30) from insects and mammals, including also the human regucalcin sequence [37]. Based on the genomic structure, it had been proposed that the luciferin-regenerating enzyme/SMP30 existed prior to the invertebrate/vertebrate bifurcation [37].

In the present study, in addition to the presence of the gene encoding the luciferase, we also report on the existence of a gene for the luciferin-regenerating enzyme. Furthermore, after having demonstrated for the first time both the property of the demosponge spicules acting as an optical waveguide and the capacity of the animals to produce light in crude extracts, we show that the recombinant luciferase is bioactive and causes bioluminescence activity *in vitro*. Finally it is shown that the level of expression of the luciferase gene is strongly down-regulated if cells from *S. domuncula* are kept in the light. For those studies primmorphs, a special form of 3D-cell culture [38], were applied.

Materials and methods

Chemicals, materials and enzymes. The restriction enzymes, the Total RNA Isolation Kit as well as the *Escherichia coli* expression system with Gateway-Technology and Ni-NTA Agarose were purchased from Invitrogen (Carlsbad, CA; USA); rotenone, 4',6-diamino-2-phenylindol (*DAPI*), ATP, coenzyme A, Na-silicate solution, sterile seawater and *P. pyralis* recombinant firefly luciferase from Sigma (St. Louis, MO; USA); *TriplEx2* vectors (Clontech, Palo Alto, CA) and BugBuster Reagent from Novagen (Darmstadt; Germany); polyvinylidene fluoride (PVDF)-immobilon membranes (Millipore-Roth, Karlsruhe, Germany); Cy3-conjugated $F(ab')_2$ goat anti-rabbit IgG from Jackson ImmunoResearch (Cambridgshire; UK); BM chemiluminescence blotting substrate kit, proteinase inhibitor cocktail and CDP (disodium 2-chloro-5-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro)-tricyclo [3.3.1.1^{3,7}] decan}-4-yl)phenyl phosphate) from Roche (Mannheim; Germany); rhodamine-conjugated goat anti-rabbit immunoglobulin Dako (Carpinteria; USA); L-arabinose and HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] from Roth (Karlsruhe; Germany); and the D-luciferin free acid AppliChem (Darmstadt; Germany).

Sponges and primmorphs. Specimens of the marine sponge *Suberites domuncula* (Porifera, Demospongiae, Hadromerida) were collected in the Northern Adriatic near Rovinj (Croatia), and then kept in aquaria in Mainz (Germany) at a temperature of 17 °C for more than five months.

Primmorphs were obtained from single cells as described before [38]. They were cultivated in natural seawater, supplemented with 0.2 % RPMI1640 medium [39] at 16 °C (± 1 °C). The incubation was performed in an incubation chamber (Liebherr, Biberach; Germany (type 20011)). They reached sizes of 5–7 mm after five days. Then the primmorphs were transferred to a silicate cushion that had been prepared from orthosilicate in sterile six- well plates

(Nunc, Langenselbold; Germany). In detail, 1 ml of water glass (Na-silicate solution) containing 27% SiO_2 and 14% NaOH was supplemented with sterile HEPES buffer (1 M; pH 7.1) in a dilution 1:10 (v/v) to reach pH of 8.3; during this reaction the silicate precipitated to a cushion. Subsequently, the gel/solid material was left for 1 h at room temperature prior to three washing steps with sterile seawater. The primmorphs (after five days) were transferred to this silicate cushion and the incubation proceeded for three days either in complete darkness or under illumination with 50 lux with a Lumi-Lux-840 (4000 °K) lamp from Osram (München; Germany).

For the analysis of the optical properties of the siliceous spicules, both *S. domuncula* skeletal elements, and *Monorhaphis chuni* (Porifera, Hexactinellida, Amphidiscosida, Monorhaphididae) giant basal spicules have been used. *S. domuncula* forms only macroscleres, to be classified as oxeas and styles (in the minority) (length 50–350 µm and diameter of 4–6 µm) or tylostyles (in the majority) (90–320 µm x 5–8 µm). They had been obtained from tissue by treatment first with sulfuric acid/nitric acid and then with n-butanol/water/NaDodSO4 [40]. *M. chuni* giant basal spicules had been cleaned from adhering tissue also with sulfuric/nitric acid, as described [40].

Light transmission studies. For the exposure of giant basal spicules (*Monorhaphis*) to light (a part 6 cm in length and 4 mm in diameter; or a complete spicule 80 cm x 4–5 mm) the skeletal elements were fixed and exposed, at the end with the largest diameter, to a white light source (AQ-4303B from Ando Electronics, Kawasaki; Japan) with a spectrum ranging from 400 nm to > 1600 nm. The light of a halogen lamp was coupled into a multimode fiber with an adapted collimator, which delivered a nearly parallel beam of ≈4 mm in diameter behind the exit aperture, as described [11]. The transmitted light was collected with a collimator into a second multimode fiber for analysis within an optical spectrum analyzer (AQ-6315A from Ando Electronics, Kawasaki; Japan).

For the demonstration that the *S. domuncula* tylostyles can also act as optical waveguides, the spicules (150 µm x 6 µm) were exposed to a white light source under the VHX-100 Digital Microscope (Keyence, Neu-Isenburg; Germany), equipped with a VH-Z100 zoom lens (magnification from 100x to 1000x). The intensity of the light (400 to 900 nm) was 6 klx; the beam had been coupled with the spicule in the same orientation as its longest axis.

Bioluminescent flashing by sponge tissue. Sponge specimens were adapted to complete darkness for a period of three days. During that time the animals had been kept under optimal aeration and water quality conditions [41]. Tissue samples (cubes with an edge length of 2 cm) were taken and exposed with their cut surface to the Chemiluminescent Detection Film (Lumi-Film; Roche; Mannheim; Germany) in a petri dish (diameter 5 cm; height 0.4 cm). The borders between the water phase and the film were sealed with a silicone O-ring. Then the setup was turned around and the exposure time of the tissue to the film was set to 24 h. As a control, a tissue sample was used that had been preincubated for two days in a medium supplemented with rotenone (1 µM) to inhibit the mitochondrial respiratory chain complex I [42, 43] and to reduce the metabolic activity of the cells.

Molecular cloning of luciferase gene from S. domuncula. A fragment (EST database accession no. s3631) of the sequence *SDLUC* was identified in the sponge database from *S. domuncula.* The sequence which comprises 795 nucleotides (nts) spanned the open reading frame (ORF) of the sponge luciferase fragment between aa_{303} and aa_{549} of the complete sequence. The technique of polymerase chain reaction (PCR) was applied to identify the complete cDNA coding for luciferase gene (LUC) from *S. domuncula.* Insert-specific primers were designed (SD_FLUC: 5′-AGGATGAGGGAGTCAATGAG-3' and SD_RLUC: 5'-GAGGTGCAGTGTTCACTCGAT-3') and PCR reactions performed with the vector primers of the clone in order to complete the *SDLUC* sequence. PCR conditions were as follows: 95 °C for 3 min, followed by 35 amplification cycles at 95 °C for 30 s, 56 °C or 58 °C for 30 s, 74 °C for 70 s and a final extension step at 74 °C for 10 min. Fragments were isolated and cloned into the *pGEMT* vector in *E. coli* TOP10 cells (Invitrogen). Sequencing was performed with primers directed to the SP6 promoter and the T7 promoter. The complete clone (*SDLUC*) encoding the protein LUC_SUBDO is 1743 nts long (excluding the poly (A) tail). The complete cDNA was termed *SDLUC* and its deduced protein LUC_SUBDO.

Molecular cloning of luciferin-regenerating enzyme (LRE_SUBDO). A fragment (EST database no. sd002_005d) of the putative sequence *SDLRE* was identified in the sponge database from *S. domuncula.* The sequence which comprises 720 nts spanned the ORF of the putative luciferin-regenerating enzyme between aa_3 and aa_{245}. To complete the cDNA, two primers (SD_F1LRE: 5'-CACTGATATTGAAGGTCGTTTG-3' and SD_R1LRE: 5'-GTAGGACTAGGATTAAGACC-3') were designed based on the fragment. For amplification through PCR these two primers, in combination with the vector primers, had been applied. The conditions were; initial denatura-

tion at 95 °C for 5 min, 35 amplification cycles at 95 °C for 30 s, 56 °C or 58 °C for 35 s, 74 °C for 40 s and the final extension step at 74 °C for 10 min. Sequencing had been performed as described above. The complete clone *SDLRE* is 918 nts long (excluding the poly(A) tail); the deduced protein was termed LRE_-SUBDO.

Sequence analyses. The sequences were analyzed with computer programs BLAST (2005; http://www.ncbi.nlm.nih.gov/blast/blast.cgi) and FASTA (2005; http://www.ebi.ac.uk/fasta33/). Multiple alignments were performed with CLUSTALW Ver. 1.6 [44]. Phylogenetic trees were constructed on the base of aa sequence alignments by neighbour-joining, as implemented in the "Neighbor" program from the PHYLIP package [45]. The distance matrices were calculated using the Dayhoff PAM matrix model as described [46]. The degree of support for internal branches was further assessed by bootstrapping [45]. The graphic presentations were prepared with GeneDoc [47].

Preparation of recombinant S. domuncula luciferase and of antibodies. The complete ORF (from the start until the stop codon) of *SDLUC* was expressed in *E. coli* using the Gateway-Technology in the *pDEST17* vector as described [48, 49]. The clone *SDLUC* was first inserted into the donor vector *pDONR*221 after amplification via two primers directed against nt_1 to nt_{24} of the luciferase cDNA (*SDLUC*) and nt_{1726} to nt_{1746} at an initial denaturation at 95 °C for 3 min, followed by 35 amplification cycles at 95 °C for 30 s, 66 °C for 35 s, 72 °C for 2 min 15 s, with a decreasing temperature of 0.1 °C in every cycle, and a final extension step at 72 °C for 10 min. After insertion in the expression vector *pDEST*17, the clone SDLUC was expressed in the *E. coli* host strain BL21-AI (Invitrogen), growing in LB medium with 50 µg/ml carbenicillin, in the presence of 0.2 % (w/v) L-arabinose for up to 12 h at 20 °C. The bacterial pellet was then lysed with BugBuster (primary amine-free) "Protein Extraction Reagent" and 1 µl/ml of benzonase (Novagen) together with the protease inhibitor cocktail (Roche) for 1 h at 20 °C; according to the instructions of the manufacturer (Novagen/Merck KGaA, Darmstadt; Germany). After sonication on ice 3 x 15 s (with 15 s cooling periods in between each), the lysate was centrifuged for 30 min at 10 000 rpm at 4 °C. The insoluble fraction obtained was solubilized with the lysis buffer (50 mM KH_2PO_4; pH 8.0, 6 M urea, 300 mM KCl and 5 mM imidazole). After sonication on ice 3 x 15 s (with 15 s cooling periods in between each), the suspension was centrifuged 30 min at 10 000 rpm at 4 °C and the polyhistidine-tagged protein was purified by Ni-NTA Agarose affinity chromatography, according to the instruction for preparing buffers for purification of polyhistidine-tagged proteins (Macherey-Nagel). The size of the protein was determined to be 67 kDa (exact: 66.5 kDa, inclusive of the His-tag). The recombinant protein was termed rLUC_SUBDO.

Preparation of antibodies. The ORF of *SDLUC* was expressed in *E. coli* (see above) and the recombinant, purified protein (rLUC_SUBDO) was used for the production of polyclonal antibodies (PoAb), as described [40, 50]. The antibodies were raised in female rabbits (White New Zealand); 10 µg of recombinant protein per injection were dissolved in PBS (phosphate-buffered-saline). After three boosts the serum was collected; the PoAb against luciferase was termed PoAb-aLUCSUBDO. The titer of the antibodies was 1:10 000. In controls, adsorbed PoAb-aLUCSUBDO (100 µl of antibodies were incubated with 20 µg of rLUC_SUBDO) was used. This antibody preparation did not result in a specific antigen-antibody complex formation.

$NaDodSO_4$-PAGE analysis. Na-dodecyl sulphate polyacrylamide gel electrophoresis ($NaDodSO_4$-PAGE) was routinely performed as follows. Samples of about 5 µg protein were dissolved in loading buffer (Roti-Load; Roth, Karlsruhe, Germany), boiled for 5 min, subjected to $NaDodSO_4$-PAGE (10 % polyacrylamide and 0.1 % $NaDodSO_4$), and electrophoresed as described [51]. The gels were stained with Coomassie brilliant blue. The protein size standard "Dual Color" (Roth) was used to estimate the size of the proteins.

Western blot analysis. Size-separated proteins were transferred to PVDF-Immobilon membranes according to Kyhse-Andersen [52]. Membranes were saturated with 5 % non-fat dry milk, 1.5 % bovine serum albumin [BSA] in TBS-T buffer (20 mM Tris-HCl; pH 7.6, 137 mM NaCl, 0.1 % Tween-20) at room temperature. PVDF membranes were then rinsed in TBS-T and incubated for 1 h with polyclonal antibodies (PoAb-aLUCSUBDO; 1:3000 dilution) diluted in TBS-T, supplemented with 5 % non-fat dry milk and 1.5 % BSA. Membranes were washed three times in TBS-T and then incubated for 1 h with anti-rabbit IgG (alkaline phosphatase conjugate; 1:4000 dilution). The immunocomplexes were visualized with the color develop system NBT/BCIP (Roth).

Luminescence measurement. Luminescence assay was performed according to Michel et al. [53] and Oba et al. [22]. The standard reaction mixture (100 µl) contained 0.25 mM D-luciferin (free acid), 5 mM ATP, 0.3 mM coenzyme A (CoA), and 5 mM $MgCl_2$ in a 0.4

M Tris-HCl buffer (pH 8.0). The reaction was started by the addition of the purified, recombinant luciferase (0.5 µg or 3 µg) at 22 °C. The light intensity was detected with a luminometer "Lumat LB 9501" (Berthold Detection System, Bad Wildbad; Germany), detecting emitted light between 390 and 620 nm. The activity of the enzyme is given in relative light units (RLU), by integration of the counts determined for the initial 60 s. A series of six parallel assays were performed and the mean values were calculated (± SD) according to Sachs [54]. As a control the recombinant luciferase was heated (95 °C for 5 min) prior to the addition to the assay. In parallel, the bioluminescence emission spectrum for the recombinant luciferase was determined on a Perkin-Elmer LS55 fluorescence spectrophotometer with the excitation light source shunted under an optimized experimental set up (gate/delay times, scan rate). Data were corrected for the spectral sensitivity of the photomultiplier tube.

In one further series of experiments tissue from *S. domuncula* was extracted with four parts of a 0.4 M Tris-HCl-buffer (pH 8.0; supplemented with 50 mM NaCl, 0.5 mM EDTA and 5 mM $MgCl_2$). After grinding, the suspension was stirred at 0 °C for 1 h and subsequently, centrifuged (20 000 x g). The clear supernatant was collected and 10 µg of protein were added to the assay. The incubation and recording conditions were the same as for the recombinant protein.

Histological analysis. Sponge tissue was fixed in paraformaldehyde, embedded in Technovit 8100 (Heraeus Kulzer, Wehrheim; Germany), and sliced, essentially as previously described [55, 56]. Since the spicules had not been removed from the tissue only smaller tissue sections could be made. The 8 µm-thick slices were incubated with PoAb-aLUCSUBDO (1:3000 dilution) overnight. Then the slides were treated with Cy3-conjugated goat anti-rabbit immunoglobulin G (IgG) for 2 h. The pre-immune rabbit serum was used as a control. In parallel, slices were stained with 5 µg/ml of DAPI for 30 min to identify the DNA within the nucleus. The slices were inspected with an Olympus AHBT3 microscope; the unstained slices were checked directly using Nomarsky interference contrast optics. In addition, the sections were inspected under immunofluorescence light at an excitation light wave-length of 546 nm for the identification of Cy3-stained structures (490 nm for DAPI).

Northern blotting. RNA was extracted from liquid-nitrogen pulverized sponge tissue with TRIzol Reagent. Total RNA (5 µg) was electrophoresed through 1% formaldehyde/agarose gel and blotted onto Hybond N^+ membrane following the manufacturer's instructions [57]. Hybridization was performed with the *SDLUC* probe, comprising a 472 nts long part of the cDNA, or the house-keeping cDNA for β-tubulin [*SDTUB* (AJ5508069)], as described [58]. Northern blot signals were quantified by the chemiluminescence procedure [59] with CDP-Star as substrate (Roche). The screen was scanned with the GS-525 Molecular Imager (Bio-Rad; Hercules, CA; USA).

Further analytical method. For the quantification of protein the Bradford method (Roti-Quant solution – Roth) was used [60].

Results

Light transmission through spicules. The property of the siliceous spicules of sponges has been well established using the large spicules from hexactinellids, primarily from *Euplectella aspergillum* [10], *Hyalonema sieboldi* [11] and *Monorhaphis* [12, 61]. It is especially the giant basal spicule from *Monorhaphis* which allows analyses of the optophysical properties in an exemplary manner. When such an 80 cm long spicule was coupled in a free-space way with a white light source (Fig. 1B) and exposed to light between the wavelength range of 400 nm and 1600 nm, a distinct cut-off of the wavelengths below 600 nm and above 1400 nm was measured, as described earlier [12]). During the passage of the light, evidenced by partial photon scattering outside the spicule waveguide (Fig. 1D), a distinct white-to-red color shift along the spicules of glass sponges is seen, in agreement with the predominant optical absorption of the lower-wavelength components (< 600 nm) of the visible spectrum. Until now the optical waveguide function of a demosponge spicule had not been demonstrated. For our studies we used the 150 µm long tylostyles from *S. domuncula.* Those spicules were illuminated under a light microscope with a power of 6 klx at a spectral range between 400 to 900 nm (Fig. 1A). In contrast to the optical properties of the giant basal spicules from *Monorhaphis*, the light quality (white) did not change during the course through the shorter spicules (Fig. 1C).

***Bioluminescent* flashing of sponge tissue.** The first evidence that *S. domuncula* has the ability to flash came from an exposure study using tissue from a dark-adapted animal. If a tissue sample (Fig. 2A) had been contacted to a chemiluminescent detection film for 24 h, a clear signal could be detected after development with the chemiluminescent substrate (Fig. 2C).

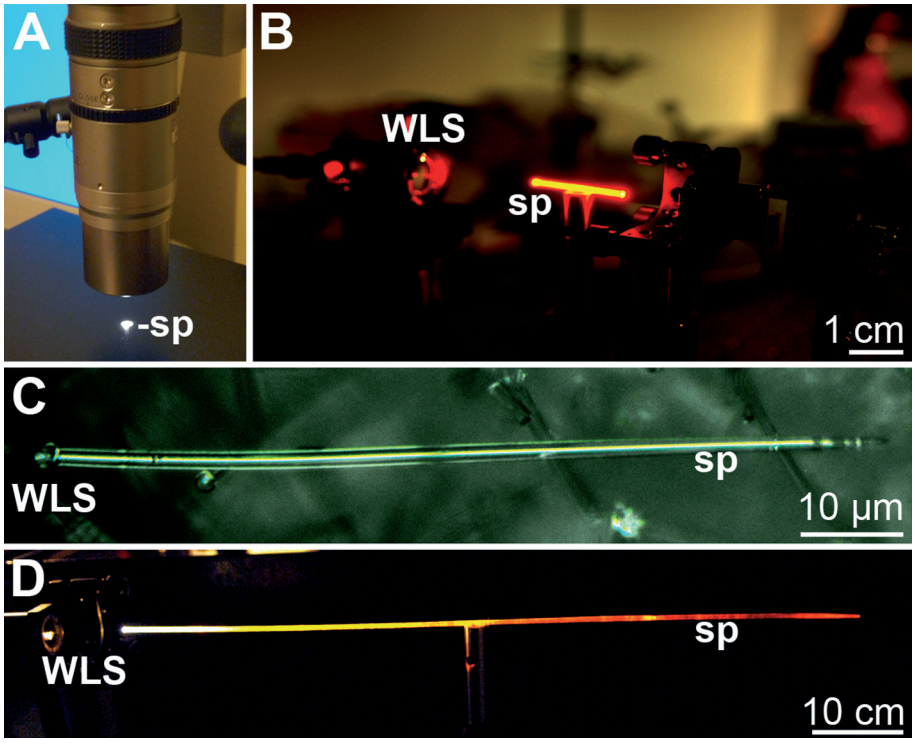

Figure 1. Transmission properties of spicules both from the demosponge *S. domuncula* (tylostyles) and the hexactinellid Monorhaphis (giant basal spicules). (*A*) Analysis of the *S. domuncula* spicules (sp) under the VHX-100 Digital Microscope with a light spectrum between 400 to 900 nm and an intensity of 6 klx. (*B*) Giant basal spicule of *Monorhaphis* (length of the piece: 6 cm; diameter: 4 mm) as an optical fiber. The white light source (WLS) is focused through a biconvex lens onto one end of the spicule (sp), which is immobilized within a canula. (*C*) A *S. domuncula* tylostyle (150 µm x 6 µm) is illuminated with white light (WLS). During the course through the tylostyle spicule (sp) the quality of the white light did not change. (*D*) In contrast to the demosponge spicule, the color of the white light changes to red during the course through the long (80 cm x 4 mm) giant basal spicule from Monorhaphis (sp).

As a control, tissue from an animal that had been pre-treated with rotenone was exposed to the film (Fig. 2B). Under otherwise identical conditions, this tissue caused no signal on the film (Fig. 2D).

Sequence analysis of the S. domuncula luciferase cDNA. The complete cDNA was isolated (*SDLUC*); it comprises 1973 nts, as confirmed by Northern blot analysis (2.3 kb (see below)). One ORF could be deduced from $nt_{1-3(Met)}$ to $nt_{1744-1746(stop)}$, encoding a 581aa long polypeptide (LUC_SUBDO) (Fig. 3A), from which a predicted molecular weight of 63 968 Da (isoelectric point 6.4) can be calculated. This is close to the size of the luciferase protein from *P. pyralis* [62]. LUC_SUBDO shares highest sequence similarity to luciferase from *P. pyralis* (CAA59281; 49 % similar residues). Lower are the similarities to the 4-coumarate-CoA ligases and the acetyl CoA synthetases, with only 36 % similarity (19 % identity) to the acetyl CoA synthetase from *Saccharomyces cerevisiae.* The deduced protein comprises the following characteristic regions (Fig. 3A); the AMP-binding pattern at aa_{234} to aa_{245} (ExPASy accession number: AC PS00455) and the acetyl-coenzyme A synthetase (EC 6.2.1.1) characteristic region from aa_1 to aa_{581} with the high "Expect value [*E*]" [49] of $6.3e^{-05}$. In addition, the (putative) binding sites for the substrate luciferin, based of similarities to the *P. pyralis* firefly luciferase, are highlighted (Fig. 3A). Within the major stretch of binding sites of luciferase for luciferin, aa_{379} to aa_{386},

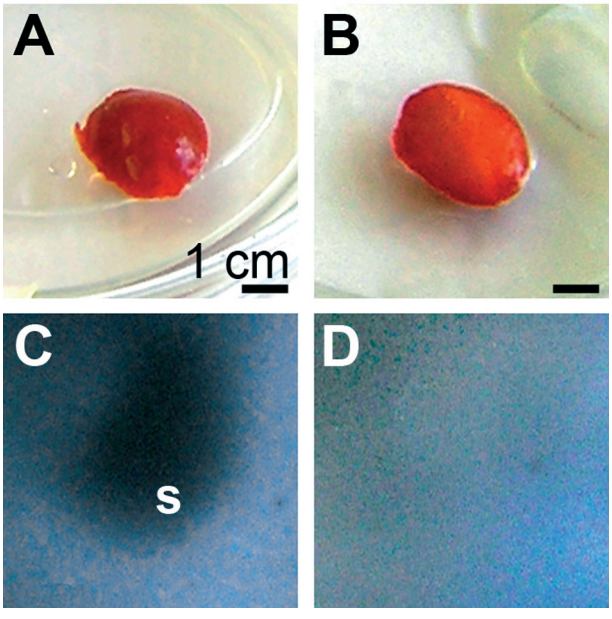

Figure 2. Generation of light (bioluminescent flashing) by tissue from *S. domuncula*, detectable by a chemiluminescent detection film, as outlined under "Materials and methods". Cubes of a dark-adapted, non-treated animals (*A*), as well as from an animal that had been pre-incubated with rotenone to suppress mitochondrial ATP generation (*B*) had been exposed to the detection film. A dark spot(s) on the film could be resolved after the development with X-ray films (*C*). In contrast, no signal had been caused by rotenone-tissue (*D*).

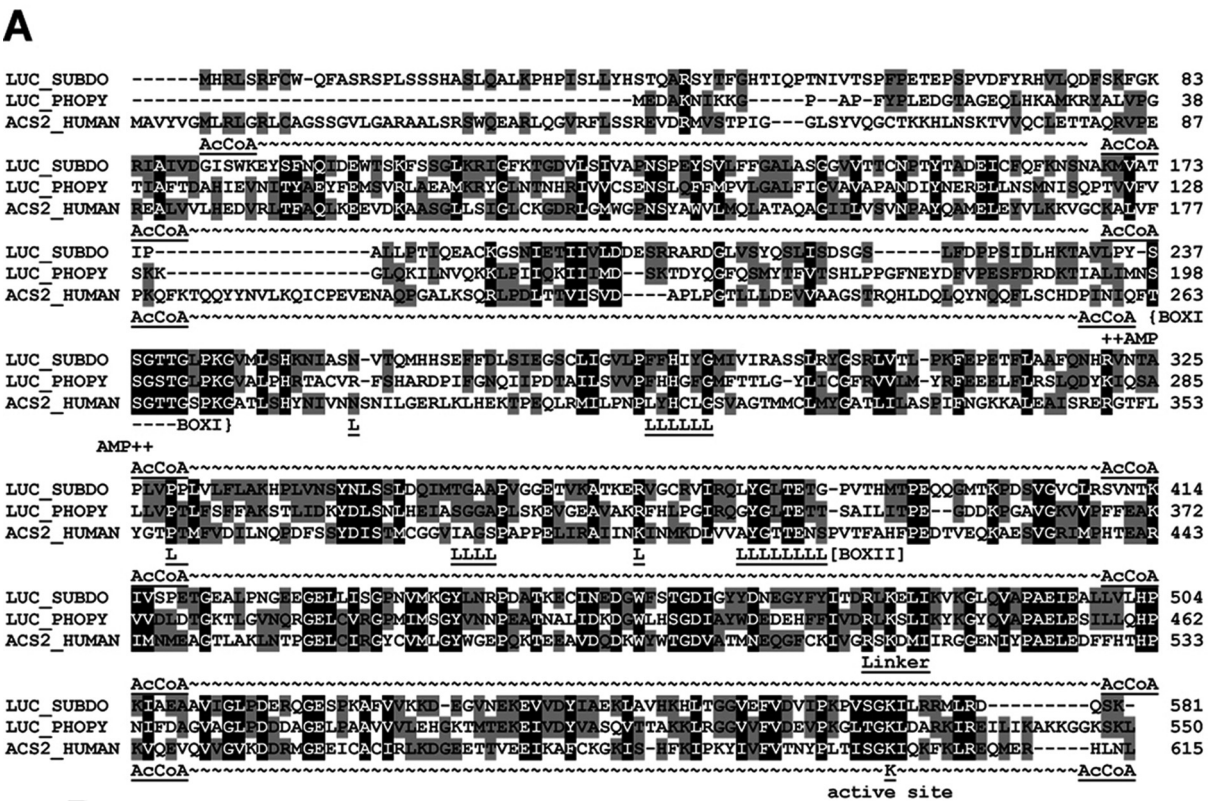

Figure 3. The *S. domuncula* luciferase polypeptide (LUC_SUBDO), deduced from the cDNA (SDLUC). (*A*) This deduced protein (LUC_SUBDO) was aligned with the related luciferase from *P. pyralis* (LUC_PHOPY; CAA59281) and the human acyl-CoA synthetase family member 2 (ACS2_HUMAN; AAH14123). The characteristic AMP-binding pattern (AMP) and the acetyl-coenzyme A synthetase region (AcCoA) are indicated and also the binding sites for the substrate luciferin between the conserved two regions (BoxI and BoxII) are highlighted. In addition, flanking luciferin-binding residues are marked (L). Finally, the flexible linker loop between the large amino-terminal domain and a small carboxy-terminal domain is marked together with the Lys-570 (K) required for the orientation of the substrate. Residues conserved (identical or similar with respect to their physico-chemical properties) in all sequences are shown in white on black; those which share similarity between two sequences are in black on grey.

BoxII exists, which had been determined to be crucial for the activity of the insect luciferases [25, 26, 63]. The corresponding BoxI, involving also the AMP-binding pattern [25], has been considered to display a role in the catalysis of the enzyme [64]. In addition, based on crystal structure analyses [29] it is established that the overall structure of the insect luciferase comprises a large amino-terminal domain and a small carboxy-terminal domain that are connected by a flexible linker loop; these linker regions are found in the *S. domuncula* enzyme between residues 479–484 (Fig. 3A). Finally, the amino acid Lys (position) 570 is mentioned, since this residue is critical for an effective substrate orientation [65].

The 15 (putative) luciferase sequences, found in the databases, were aligned with representative sequences coding for acyl-CoA synthetases and coumarate-CoA ligases, and subsequently their phylogenetic relationships were computed. The tree was rooted with the plant coumarate-CoA ligase from *Arabidopsis thaliana* as an outgroup (Fig. 3B). The tree shows that the plant coumarate-CoA ligase gives rise to one branch together with the other sequences from this class of enzymes. This branch forms the basis for the branch that also includes the acyl-CoA synthetases as well as the (putative) luciferases.

Expression of sponge luciferase. The complete ORF of the cDNA, coding for the sponge luciferase, was expressed in *E. coli.* A 67-kDa protein (representing the recombinant protein) was already dominant in samples 2 h after induction with L-arabinose after size fractionation with $NaDodSO_4$-PAGE. The intensity of this band increased until 6 to 12 h after induction (not shown). After this period the bacterial culture was harvested and the bulk of the recombinant protein (rLUC_SUBDO) was prepared. Subsequently, the rLUC_SUBDO was purified by Ni-NTA Agarose affinity chromatography, resulting in one band (67-kDa protein) on the gel (Fig. 4 lane a). Using such a protein sample, polyclonal antibodies (PoAb-aLUC-SUBDO) were prepared.

The PoAb-aLUCSUBDO recognized the 67-kDa recombinant protein (comprising the fusion protein

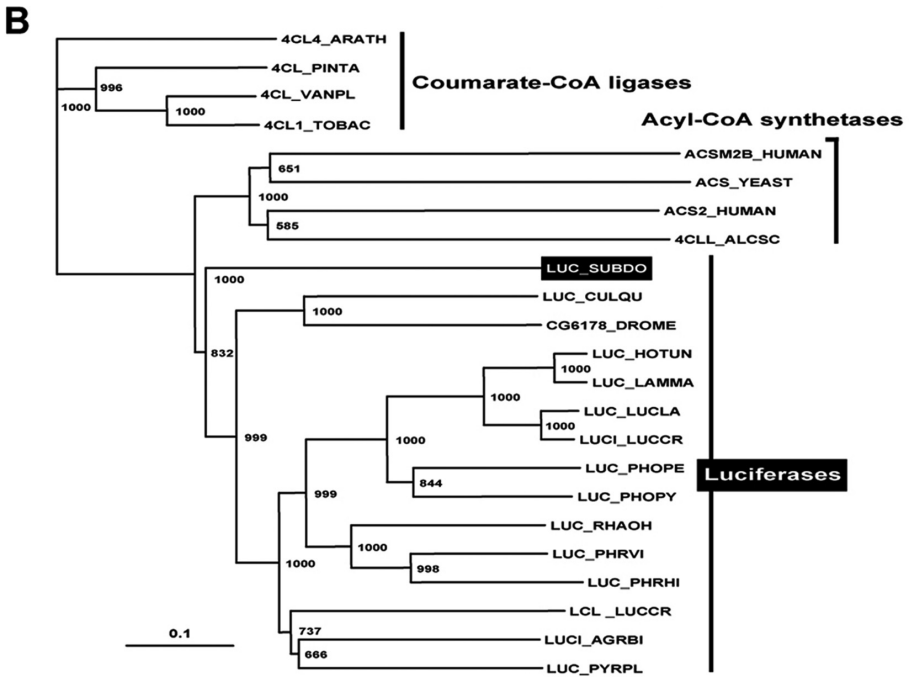

Figure 3. (continued) (*B*) These three proteins were compared with the related luciferases from the fireflies *Hotaria unmunsana* (LUC_HOTUN; AAN40975), *Luciola lateralis* (LUC_LUCLA; AAN73267), *Lampyroidea maculata* (LUC_LAMMA; AAZ74651), *Photuris pennsylvanica* (LUC_PHOPE; BAA05005), *Rhagophthalmus ohbai* (LUC_RHAOH; BAF34360), the luciferin 4-monooxygenase from the Japanese firefly *Luciola cruciata* (LUCI_LUCCR; P13129), the beetle *Pyrophorus plagiophthalamus* (LUC_PYRPL; AAQ11712), the railroad-worms *Phrixothrix vivianii* (LUC_PHRVI; AAD34542) and *Phrixothrix hirtus* (red-bioluminescence eliciting luciferase) (LUC_PHRHI; AAD34543), the mosquito *Culex quinquefasciatus* (luciferin 4-monooxygenase) (LUC_CULQU; EDS40370), the luciferase homologue from *Agrypnus binodulus* (LUCI_AGRBI; BAF96580), the paralogous genes of the Japanese firefly *Luciola cruciata* (LCL_LUCCR; BAE80729) as well as the hypothetical gene from *Drosophila melanogaster* (CG6178_DROME; NP_651221). In addition, members of the class of 4-coumarate-CoA ligase (4-coumaroyl-CoA synthase) from the *Vanilla planifolia* (4CL_VANPL; O24540), the loblolly pine *Pinus taeda* (4CL_PINTA; P41636), the common tobacco *Nicotiana tabacum* (4CL1_TOBAC; O24145) and *Arabidopsis thaliana* (4CL4_ARATH; Q9LU36), as well as the termed 4-chlorobenzoyl CoA ligase from *Alcaligenes sp.* AL3007 (4CLL_ALCSC; 1T5H AAN10109), the human acyl-CoA synthetase medium-chain family member 2B (CSM2B_HUMAN; Q68CK6) and the acetyl CoA synthetase of *Saccharomyces cerevisiae* (ACS_YEAST; EDN59709) have been included. The tree was calculated and rooted with the plant sequence from A. thaliana as outgroup. Scale bar indicates an evolutionary distance of 0.1 aa substitutions per position in the sequence.

with 63 968 Da for luciferase and 2600 Da for the His-tag) after separation of the recombinant protein by $NaDodSO_4$-PAGE and after transfer on Western blots (Fig. 4 lane a). This protein did not cross-react with a pre-immune serum (Fig. 4 lane b).

Determination of activity of recombinant luciferase. Luciferase activity was determined in the standard reaction assay, as described under "Materials and methods". In the absence of the recombinant luciferase (rLUC_SUBDO) only a few counts (RLU) could be determined in the luminometer with 920 RLU. Similarly low were the countings when ATP or coenzyme A (CoA) was omitted from the reaction assay. However, in the presence of the recombinant protein the values increased significantly (Fig. 5A). Hence, a specific activity of 72 000 RLU/0.5 µg protein can be calculated. Studies with the recombinant luciferase from *P. pyralis* were performed in parallel; under otherwise identical conditions this enzyme showed a specific activity of 346 000 RLU/µg. This series of experiments demonstrated that the cDNA cloned from *S. domuncula* encodes a luciferase, an enzyme which depends on ATP and CoA, and which generates light. In a control assay, the complete reaction mixture was supplemented with an enzyme (rLUC_SUBDO) that had been pretreated with heat. In that assay almost no luminescence activity could be recorded. The heat-sensitivity of the insect luciferase is well established [23]. Considering earlier findings that the firefly luciferase reaction is pH-dependent [21], we studied the bioluminescence generating reaction, using recombinant enzyme from *S. domuncula*, under different pH conditions (routinely the assay was performed at pH 8.0 (0.4 M Tris-HCl buffer) revealing a specific activity of 72 000 RLU/0.5 µg protein). At pH 9.0 the activity increased to 83 000 RLU/0.5 µg, while at higher or lower pH conditions the specific activities decreased; at pH 10.0 or at pH 7.0 activities of 59 000 RLU/0.5 µg or 51 000 RLU/0.5

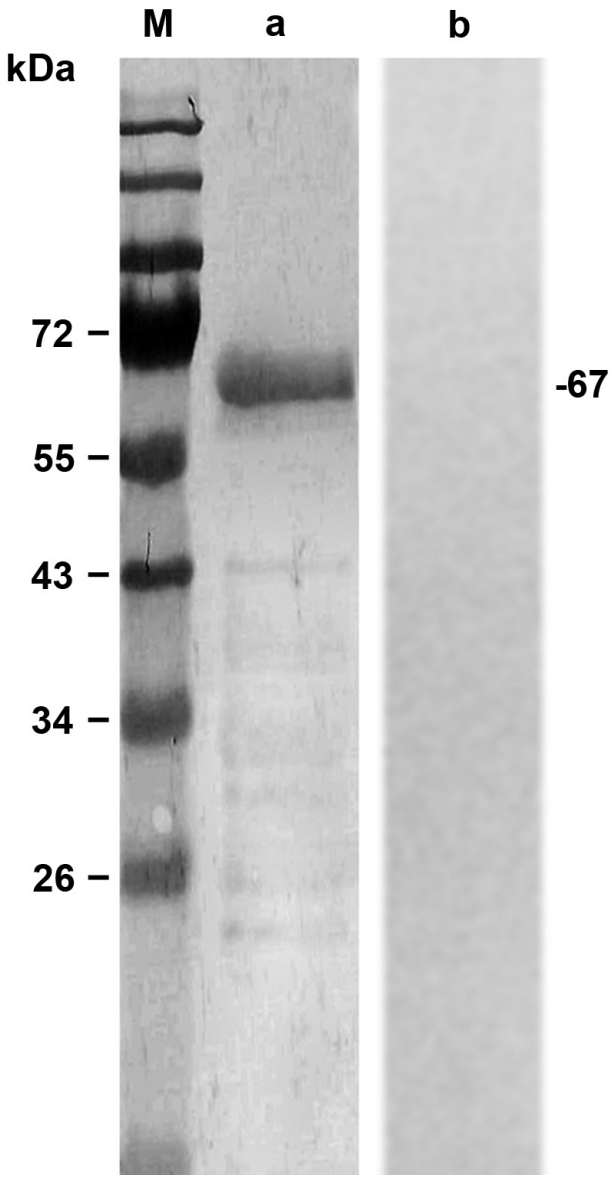

Figure 4. Preparation of recombinant luciferase. *E. coli* were transformed with the luciferase cDNA SDLUC, as described under "Materials and methods". After purification of the recombinant protein a Western blot analysis was performed. Polyclonal antibodies have been raised against the purified luciferase. (Lane a) This antiserum recognized the 67 kDa recombinant protein, while (lane b) the pre-immune serum did not recognize this protein after size separation by NaDodSO4-PAGE. M: size markers.

μg, respectively, were measured. The emission spectrum of the *S. domuncula* enzyme revealed a maximum at 548 nm and a minor peak at 590 nm (pH 8.0) (Fig. 5B), concurrent with the bioluminescence spectra of the firefly luciferase/luciferin system that generally vary in the range of 520 nm to 620 nm. So far we cannot explain why an increase of enzyme from 0.5 to 3.0 μg elicits only a doubling of RLU (72 000 and 150 000, respectively). This might be due to impurities in the enzyme preparation with potential detrimental effects. However, application of lower enzyme concentrations (0.1 and 0.05 μg) caused a proportional decrease as expected (22 000 and 12 000 RLU, respectively).

In one series of experiments a crude extract had been prepared from tissue of *S. domuncula*, as described under "Materials and methods". An aliquot of 10 μg of protein had been added to the standard incubation assay, supplemented with luciferase, ATP and CoA. Also, this sample displayed luciferase (bioluminescence) activity, with 25 300 ± 3200 RLU ($n = 5$). In controls, after omission of ATP and CoA only small quantities of bioactivity could be measured; RLU $<$ 1000.

Immunolocalization of the luciferase in sponge tissue. Antibodies (PoAb-aLUCSUBDO) which specifically recognize the recombinant protein on the Western blot were raised against the purified recombinant luciferase. These antibodies were used for the localization of the luciferase in sections, applying the technique of immunohistology. Reacting the slices with PoAb-aLUCSUBDO resulted in a bright staining of cellular structures which surround the spicules (Fig. 6C). By application of Nomarsky interference contrast optics the localization of the aquiferous canal system in the tissue (Fig. 6A) was possible: separately and by DAPI staining, the distribution of the cells (staining of the cell nuclei) (Fig. 6B) could be visualized. The specificity of the antibody preparation was assured by using pre-immune rabbit serum which did not react with any structure on the tissue slices (Fig. 6F). They had been obtained in parallel and inspected by Nomansky optics (Fig. 6D) or had been stained with DAPI (Fig. 6E).

Adaptive response of luciferase expression. Cells from *S. domuncula*, proliferating in aggregates (primmorphs), had been applied to demonstrate the steady-state expression of the *luciferase* gene (*SDLUC*). Primmorphs had been cultivated from single cells during a period of five days (Fig. 7A), prior to their transfer onto a silicate cushion (Fig. 7B). On this substrate the cells remained either in the dark for three days or were exposed for the same period to white light. Subsequently, RNA was extracted from the cells and subjected to Northern blot experiments. After size separation the gels were hybridized with the *SDLUC* probe, which resulted in a bright staining of the 2.3 kb *luciferase* transcript (Fig. 7C). A semi-quantitative analysis of the signal revealed that the level of expression was highest in primmorphs kept in the dark. Scanning of the screen showed that the expression (transcripts of *SDLUC*) in primmorphs, kept in light, was significantly lower (0.2-fold; Fig. 7C

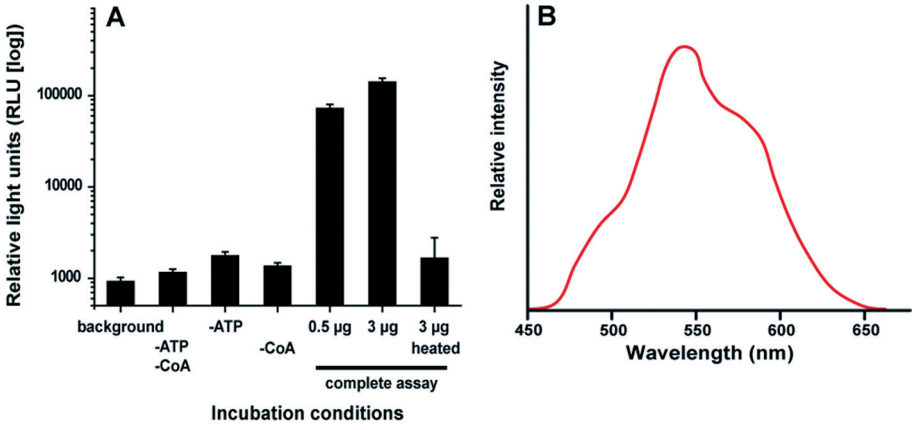

Figure 5. Determination of bioactivity of recombinant luciferase. (*A*) The *S. domuncula* luciferase cDNA was cloned and expressed and the recombinant protein assayed in the "luciferase" assay for bioluminescence. The determinations had been performed, either in the absence of ATP or CoA, or with different concentrations of recombinant luciferase, as indicated. In one series of experiments the heat-treated luciferase had been added to the reaction mixture. The generation of light was measured in a luminometer; the values are given in RLU (please notice the logarithmic scale), as indicated under Materials and methods. (*B*) Bioluminescence emission spectrum of the *S. domuncula* luciferase at pH 8.0.

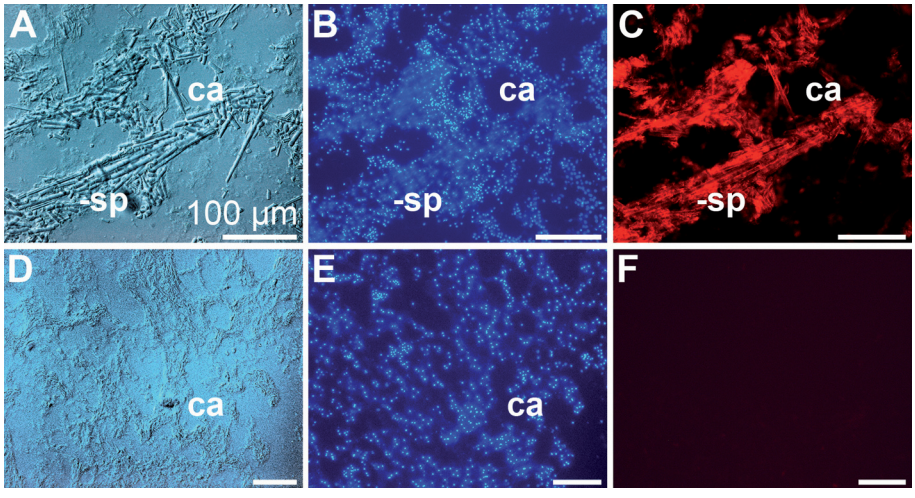

Figure 6. Localization of luciferase in tissue from *S. domuncula* by immunofluorescence staining. Tissue slices were prepared and reacted with either DAPI or antibodies (PoAb-aLUCSUBDO) that have been raised against recombinant luciferase. (*A*) Tissue section was inspected with Nomarsky interference contrast optics. The canals (ca) and the spicules (sp) can be distinguished within the tissue. (*B*) Corresponding view to visualize the cell nuclei by DAPI. (*C*) Section that had been reacted with PoAb-aLUCSUBDO and subsequently with Cy3-conjugated IgG. In a control series, a section had been inspected with interference optics (*D*) or had been incubated with pre-immune serum and then with Cy3-conjugated IgG (*F*). In addition, the section was stained with DAPI (*E*).

lane b) compared to that of cells grown in the dark (1-fold; Fig. 7C lane a). Control experiments with the housekeeping gene ß-tubulin (transcript size of 1.4 kb) showed that the same amounts of RNA had been loaded onto the gels (not shown).

Sponge luciferin-regenerating enzyme cDNA. As one consequence of the finding that luciferin can be used as substrate for the recombinant sponge luciferase we screened for the luciferin-regenerating enzyme. The cDNA from *S. domuncula* (*SDLRE*) was cloned and found to be 1146 nts long. By Northern blotting it was confirmed that this sequence was complete (1.2 kb) (not shown). The deduced protein (LRE_SUBDO) comprises 306 aa residues ($nt_{17-19(Met)}$ and $nt_{895-897(stop)}$), from which a size of 32 841 Da (isoelectric point 4.7) was calculated. The sponge sequence shows high similarity to the luciferin-regenerating enzyme from insects, with the one of *P. pyralis* as an example (49 % similar aa); Fig. 8A. The deduced protein comprises between aa_{15} to aa_{273} the characteristic SMP-30/gluconolactonase/LRE (luciferin-regenerating enzyme)-like region with an e-value of $4.3e^{-88}$ (pfam PF08450.3; [66]). The two putative luciferin/oxyluciferin binding domains [37] are found in the *S.*

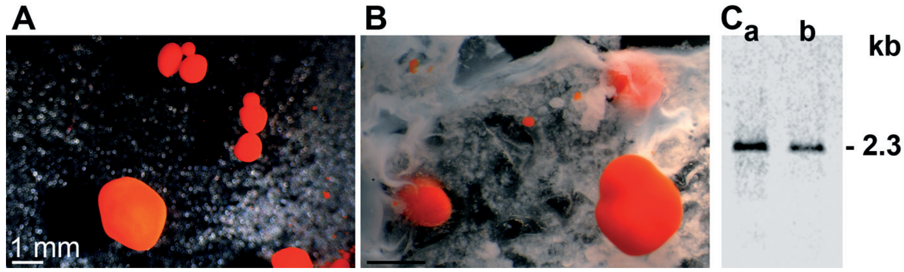

Figure 7. Adaptive response of the expression of the luciferase gene in primmorphs. Primmorphs had been prepared from single cells of *S. domuncula.* After an incubation period for five days (*A*) these aggregates were transferred to a silicate cushion (*B*) and incubated for additional three days in the dark or in the light. (*C*) RNA was extracted from both assays in the same amount (5 µg) and size separated. After blot transfer the filters were hybridized with the labeled SDLUC probe. RNA from cells growing in the dark (lane a) and those exposed to light (lane b) was analyzed.

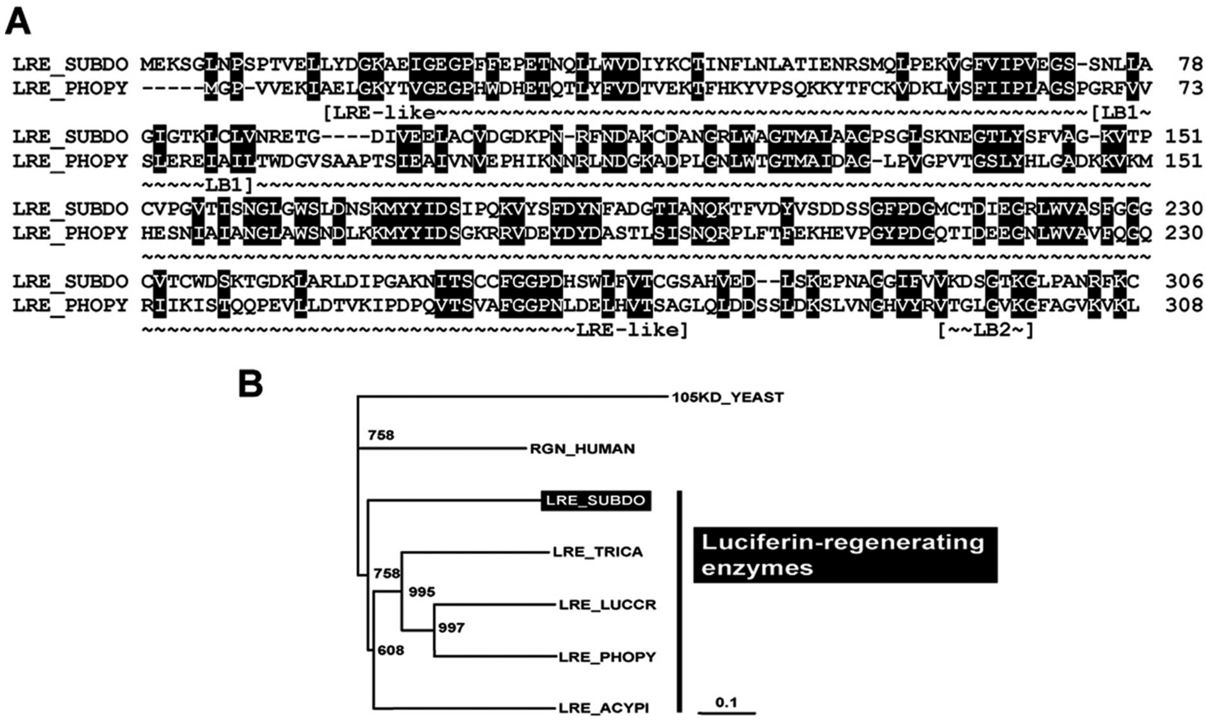

Figure 8. The deduced polypeptide of the *S. domuncula* luciferin-regenerating enzyme (LRE_SUBDO). (*A*) This sponge sequence is aligned with the luciferin-regenerating enzyme from *P. pyralis* (LRE_PHOPY; BAB60700.1). The SMP-30/gluconolactonase/LRE (LRE-Like) and the luciferase binding domain 1 and 2 (LB1 and -2) are marked. (*B*) For the construction of the phylogenetic tree these two sequences were aligned with the related luciferin-regenerating enzymes from the pea aphid *Acyrthosiphon pisum* (LRE_ACYPI; XP_001951053.1), the red flour beetle *Tribolium castaneum* (LRE_TRICA; XP_967986.1), the Genji-firefly *Luciola cruciata* (LRE_LUCCR; BAB85479.1), the human regucalcin sequence (RGN_HUMAN; BAD97080.1) and the 105 kDa protein, associated with polyadenylation factor 1 (105KD_YEAST; 190406148). The tree was computed and rooted with the yeast sequence.

domuncula sequence at aa_{74} to aa_{87} and at aa_{291} to aa_{298} (Fig. 8A).

A phylogenetic relationship was established by comparing this sponge polypeptide with the different insect luciferin-regenerating enzymes from the aphid *Acyrthosiphon pisum*, the beetle *Tribolium castaneum*, the Genji-firefly *Luciola cruciata* and the human regucalcin sequence. Regucalcin was discovered in 1978 as a Ca^{2+}-binding protein that does not contain an EF-hand motif of Ca^{2+}-binding domain, and which is involved in regulation of intracellular signaling pathways [17, 67]. Using the next similar sequence from *S. cerevisiae*, a 105 kDa protein known to be associated with the polyadenylation factor from *S. cerevisiae,* as outgroup, it can be seen that the sponge luciferin-regenerating enzyme falls in the group of those luciferin-recycling enzymes [32, 36]; Fig. 8B.

Discussion

Based on biological observations, a series of compelling evidences for the existence of a photo-reaction in sponges had been observed. As an example, it had been demonstrated that sponge embryos, larvae, and adult sponges react to light [68–70]. Even more, recent studies revealed that sponges can discriminate between light of different wavelengths [71, 72]. The strongest reaction is seen towards blue light around 440 nm and 600 nm [72]. Recently, it had been recorded that photosynthetically active microorganisms might exist in the sponge *Tethya aurantium* that generate light which is transmitted through the spicular system [73]. In discussions of the optophysical light waveguide experiments [8, 10] it was proposed that, in sponges, presumably potential microorganisms that live in symbiosis with them might generate light which is transduced and guided through the silica spicular structures. However, it can be argued that such a symbiotic system (microorganisms-sponges) is too interference-prone to maintain a sensory system that should be considered as crucial for surviving in the biotope. It is highly conceivable that the remarkable fiber-optical features of the spicules do not exist merely accidentally, since this silica-based spicular system has been kept by these animals for over 600 Ma [1].

In general, the existence of photoreception systems which do not include an eye, e.g. the non-ocular dermal photoreaction system in snails [74], or the extra-ocular perception system in the frog *Rana catesbeiana* that is required for spatial orientation [75], is not infrequently found in nature. However, all these metazoan systems involve synapses that do not exist in sponges. Based on those considerations we proposed [11], and data on the existence of the luciferase and the luciferin-regenerating enzyme gathered here are taken as experimental support, that the animals not only possess light waveguides, the spicules, but also have the potential to generate light by the luciferase that can be transmitted through the spicules.

For the sponges it is considerably energy consuming to build spicules of such purity in silica. The pureness of the sponge spicular silica reaches quartz-glass grade [19]. The spicules contain only little concentrations of cations, which provide them with an unusual hardness [76]. These animals accumulate trace concentrations of silicon (silicic acid) – existing in the marine environment – in their cells via an ATP-consuming pump [77] and deposit silicic acid first in special vacuoles [78] and finally in the spicules [79]. The presented data on the optophysical properties of *S. domuncula* spicule suggest that sponge spicules, both in demosponges and in hexactinellids, act as biological glass fibers. Focusing on hexactinellids, only a few sponge species occur in the more shallow waters, e.g. *R. racovitzae*, where their spicules might function as light-collectors [8, 9]. However, most of them live in the deep sea at depths of 1000 m and more [10–12]. Hence, light might come from symbiotic microorganisms such as *E. aspergillum*, *H. sieboldi*, or *M. chuni* or – as experimentally shown in the present study – from their own light-producing luciferase/luciferin system. In the present study this view has been extended by showing that spicules from demosponges, with *S. domuncula* as an example, can also act as optical wave guides.

The presented data unambiguously show that the demosponge *S. domuncula* is provided with genes encoding the two major enzymes involved in the well elucidated bioluminescence system from insects, the luciferase and the luciferin-regenerating enzyme. Sequence analyses show that the genomic organization as well as the presence of the poly(A)-tail in their transcribed mRNA (to be published) unambiguously indicate that these genes are of sponge origin and do not stem from bacterial contaminations. This view is additionally supported by the phylogenetic analyses of their deduced polypeptides, which clearly show a distinct relationship of the sponge luciferase and the luciferin-regenerating enzyme to the metazoan/insect luciferase system. More specifically, the sponge luciferase falls into the group of luciferases rather than into the class of the related acetyl-CoA synthetases [23]. Likewise, the sponge luciferin-regenerating enzyme comprises a high sequence similarity to the enzymes identified in insects. Previous phylogenetic analyses revealed that most of the sponge proteins, deduced from their cDNAs, share high sequence similarity with vertebrate proteins [see: 80], with distinct exceptions such as some invertebrate-related proteins involved in immune response, like tachylectin [81], or as here, with the luciferase.

The recombinant sponge luciferase produced in *E. coli* is bioactive, depends on ATP and on CoA, is heat-labile, and displays a bioluminescence emission in the range of 490 nm to 620 nm. All these parameters are in common with hitherto prepared insect luciferases [see: 82]. CoA had been proven to act as a "substrate"-like component in the reaction, as known from recent studies [83]. This part of our study is corroborated also by the described experiment highlighting that the tissue from *S. domuncula* and its extracts produce light signals that can be detected by sensitive films in the dark.

Immunohistological studies show that antibodies raised against the recombinant sponge luciferase recognize very bright areas within the tissue which

are rich in spicules, while pre-immune serum did not. This result is likewise suggestive of an association of the luciferase with spicules in *S. domuncula*. However, here further studies have to be performed to clarify and elucidate the coupling between the light-generating luciferase system and the (potential) inorganic silica waveguide. At present we are working on the photo-pigment system in *S. domuncula* with carotene at the focus. This pigment is known to be involved in light reception in general [84] and also in invertebrates in particular [85]. Besides a series of carotene-derivatives, we also identified/cloned the two key enzymes in *S. domuncula* involved in the catabolic pathway of carotene, such as the β-carotene-oxygenase and the retinal dehydrogenase. After this we will approach the pigment-binding proteins.

One further clue additionally supporting our conclusion that the luciferase/luciferin system in sponges has a role in bioluminescence came from adaptation studies using primmorphs which had been exposed for three days to light. During that period the steady-state expression of the luciferase gene was significantly down-regulated, suggesting that under light conditions the luciferase system is not required or is even not advantageous for the proposed coordination system.

Taken together, the studies reported here demonstrate that in the demosponge *S. domuncula* a light-generating system might exist which functions both *in vivo* and *in vitro*, using the recombinant protein. Hence, the notion [11] that this biosensor system, composed of the organic light producing luciferase and the inorganic light transducing spicules, represents a novel photoreception system becomes progressively concrete. Studies are in progress to identify and characterize the coenzyme/substrate for the luciferase in sponge tissue. Based on the gathered data here, on the existence of a luciferin-regenerating enzyme, it is expected that a luciferin-related molecule is also used for the sponge luciferase *in vivo*.

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The sequences from *Suberites domuncula* have been deposited (EMBL/GenBank); the cDNA for luciferase (*SDLUC*; under the accession number FM201300), and for luciferin-regenerating enzyme (*SDLRE*; FM201301).

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